Response of Atlantic Salmon Eggs and Fry to Low Levels of Aromatic Hydrocarbons (AHs)

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ABSTRACT

Anderson, J. M., and V. Zitko. 2000. Response of Atlantic salmon eggs and fry to low levels of aromatic hydrocarbons (AHs). Can. Tech. Rep. Fish. Aquat. Sci. 2318: iii + 15 p.

Atlantic salmon *(Salmo salar)* eyed eggs and yolk-sac fry were exposed to low levels of aromatic hydrocarbons (AHs) obtained from diesel fuel, and from Alaskan crude oil, provided by the National Marine Fisheries Service. The latter duplicated oil spilled by the *Exxon Valdez* in 1989, to which Pink salmon *(Oncorhynchus gorbuscha)* were extremely sensitive. Atlantic salmon, at least from the eyed stage onwards, were not as sensitive to AHs from Alaskan oil as were Pink salmon, where exposure began shortly after fertilization. For Atlantic salmon, no excess mortality occurred as a result of exposure to a nominal diesel fuel concentration of 60 µg/L, but cumulative mortality amounted to 60% and 30% after exposure to sediments contaminated with 2% and 10% diesel fuel, respectively. With sediments contaminated with 2% Alaska crude oil and with artificially weathered crude oil, the mortality was about 50% and 20 %, respectively. AHs, whether from diesel fuel or from crude oil, are toxic when present in concentrations greater than 100 µg/L, as measured by ultraviolet (UV) spectrophotometry. Unlike Pink salmon, growth of Atlantic salmon fry was not affected by exposure to AHs, at least during the first 2 mo of feeding. Model experiments demonstrated a non-linear relationship between the concentration of diesel fuel in contaminated sand and AH concentration in water, and a complex kinetics of AH partitioning between diesel fuel and water, and the presence of aromatic amines and phenols in diesel fuel.

RÉSUMÉ

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Des œufs embryonnés et des larves vésiculées de saumon atlantique (*Salmo salar*) ont été exposés à de faibles concentrations d'hydrocarbures aromatiques (HA) obtenues de carburant diesel ainsi que de pétrole brut de l'Alaska fourni par le National Marine Fisheries Service. L'échantillon de pétrole brut de l'Alaska était semblable au pétrole qu'avait laissé s'écouler l'*Exxon Valdez* en 1989 et auquel le saumon rose (*Oncorhynchus gorbuscha*) était des plus sensibles. Le saumon atlantique, à tout le moins à partir du stade d'œuf embryonné, n'était pas aussi sensible aux HA du pétrole de l'Alaska que le saumon rose, pour lequel l'exposition avait débuté peu après la fertilisation. Dans le cas du saumon atlantique, il n'y a pas eu de mortalité excessive comme suite à l'exposition à une concentration nominale de carburant diesel de 60µg/L, mais la mortalité cumulative s'établissait à 60 p. 100 et à 30 p. 100 après exposition à des sédiments contaminés à 2 p. 100 et à 10 p. 100 par le carburant diesel respectivement. Dans le cas des sédiments contaminés à 2 p. 100 par le pétrole brut de l'Alaska et avec du pétrole brut altéré artificiellement, la mortalité était d'environ 50 p. 100 et de 20 p. 100 respectivement. Les HA, qu'ils proviennent de carburant diesel ou de pétrole brut, sont toxiques lorsqu'ils se trouvent en concentration plus grande que 100 µg/L, mesurée par spectrophotométrie dans l'ultraviolet (UV). Contrairement à ce qui se passe chez le saumon rose, la croissance des larves de saumon atlantique n'a pas été affectée par l'exposition aux HA, au moins au cours des deux premiers mois d'alimentation. Des expériences en laboratoire ont démontré l'existence d'une relation non linéaire entre la concentration de carburant diesel dans le sable contaminé et la concentration de HA dans l'eau et une cinétique complexe de fractionnement des HA entre le carburant diesel et l'eau et la présence d'amines aromatiques et de phénols dans le carburant diesel.

INTRODUCTION

Embryos and fry of Pink salmon *(Oncorhynchus gorbuscha)* are sensitive to aromatic hydrocarbons (AHs) (Heinz et al. 1995; Carls et al. 1996). In the context of this work, the term AHs is preferred to the term PAHs, since it includes benzene and its derivatives, which may be present in diesel fuel. The concentrations, causing mortality in eggs and fry of Pink salmon, are surprisingly low, less than 1.0 µg/L of total dissolved PAHs (Heinz et al. 1995, 1999). Also, significant long-term effects can occur, such as reduced growth rate of fry during, and well after, exposure to low levels of PAHs, as well as reduced return rate of adults initially exposed to PAHs as embryos (Carls et al. 1996; Heinz 1998). The practical significance of these findings is that the composition and concentration of AHs, used in the work referred to, correspond to what has been found on the beaches of Prince William Sound in Alaska, where Pink salmon spawn in intertidal areas several years after the beaches were contaminated by oil from the *Exxon Valdez* accident in March 1989.

Oil spills like the *Exxon Valdez* incident are not a threat to freshwater phases of Atlantic salmon *(Salmo salar)*. Nevertheless, AHs are constituents of petroleum products in general, and thus are a potential source of pollution of the salmon's freshwater habitat. It was therefore decided to investigate the response of Atlantic salmon embryos and emerging fry to AHs from diesel fuel, an ever-present source of pollution from boats, and municipal and industrial outfalls. Alaskan crude oil and artificially weathered Alaskan crude oil were included in the study for comparison.

MATERIALS AND METHODS

The experiment took place at the Atlantic Salmon Federation's facilities at Chamcook, New Brunswick, leased to the Huntsman Marine Science Centre for its Atlantic Salmon Broodstock Development Program. The eggs were laid down on November 26, 1998, and had been provided to the Huntsman by Cooke Aquaculture Ltd. The parents were of Saint John River origin, which had come from Connor Bros.' in-house breeding program. At the time the experiment began on January 13, 1999, the eyed eggs had accumulated 309.9 degree^{0}C-days of temperature.

The eggs were incubated in special, 12-in. diameter cylindrical plastic pipes fitted with screening at the bottom to hold the eggs. The incubation pipes were set in 1-m diameter holding tanks supplied with running water at 2 L/min. Water up-welled in each pipe, and drained through an outlet inside the pipe set just below its top edge. Yolk-sac fry were kept in the incubation pipes after hatching until first feeding, at which time they were removed from the pipes and given free access to the 1-m holding tank.

Ordinary commercial diesel fuel was used as one source of AHs. About 25 mL were added to water in a 23-L carboy and agitated vigorously with a mechanical stirrer for at least 30 min. The contents of the carboy were added to the tank at 2 mL/min, to provide a final PAH concentration in the holding tank of 60 µg/L (ppb), as anticipated from preliminary experiments. The carboy was changed daily.

The diesel fuel was also mixed with Baker 'Sand for flash chromatography' to give oil concentrations of 2% and 10%. The mixtures were contained in fine-mesh screening, set on the bottom screening of each incubation pipe through which the water upwelled. Ron Heinz of the National Marine Fisheries Service's Auke Bay Fisheries Laboratory in Juneau, Alaska, kindly provided samples of crude oil from the Prudoe Bay oil field, the source of the oil carried by the *Exxon Valdez*, and of the same oil, artificially weathered, to simulate the residual oil still on the beaches in Prince William Sound well after the spill incident. These samples were mixed with sand, as described for the diesel fuel, to give concentrations of 2% for each oil. The nominal percentage designations for the treatments were used to describe the results.

Water samples (300-500 mL) were taken periodically from the incubation pipes and extracted with spectrograde hexane (30 mL) on magnetic stirrers for 1 h. The hexane layer was separated, volume was adjusted according to expected AH concentration, and the UV spectrum was scanned from 200-350 nm on a Hewlett Packard Model 8452 Diode Array spectrophotometer. Absorbance at 226 nm was used to calculate the concentration of AHs. Calibration curves were prepared for each oil.

Three 1-m holding tanks were used, one each for the following treatments (the number of eggs at the start of the experiment is shown in brackets):

- Control i.e. no AHs (1223),
- nominal 60 ppb diesel fuel (1909),
- 2% (444),
- 10% (599) diesel fuel,
- 2% (423) Alaskan weathered oil, and
- 2% (430) Alaskan fresh oil.

The experimental treatments began on January 13, 1999, shortly after eyeing, except for the Alaskan oil samples, for which the treatment began on January 20. All treatments were terminated when the fry were released into their respective holding tanks at first feeding on February 26. The experiment concluded on May 11, 1999. At the end of the experiment, 30 fish were randomly selected from each of the three holding tanks and weighed.

Water temperatures averaged 8°C until first feeding, when the temperature was increased to 13°C.

In a separate, model experiment, diesel fuel-contaminated sand (10 g) was prepared to give diesel fuel concentrations of 1, 2, 5, and 10%. Distilled water (100 mL) was added and the mixture was shaken gently on a wrist-action shaker for 24 h. The UV spectrum of the aqueous phase was scanned and the process was repeated with new distilled water.

To prepare the Water Accommodated Fraction (WAF) of diesel fuel (Hokstad et al. 1999), diesel fuel (50 mL) was added to distilled water (250 mL) and the mixture was very gently stirred on a magnetic stirrer for 24 h. Samples of the aqueous phase were withdrawn after 1, 2, 3, 6, and 24 h, and their UV spectra were scanned. The remaining aqueous phase was separated, its pH was adjusted to pH=9.5 with a concentrated solution of sodium hydroxide, and the flask was gently rinsed with several volumes of hexane to remove most of the remaining diesel fuel. The alkaline solution was then extracted with hexane (3 x 50 mL) to remove any additional hydrocarbons and possibly some basic compounds. The aqueous phase was then acidified with concentrated hydrochloric acid to pH=2 and extracted with hexane to extract acids and phenols. UV spectra of the hexane extracts were scanned, the extracts were concentrated almost to dryness on a rotary evaporator, and the residues were examined by thin layer chromatography on Whatman K6F silica gel plates, developed in benzene. The plates were viewed under UV light and also sprayed with Fast Black K salt (Ojanperä et al. 1990) to detect amines and phenols.

Matlab (The MathWorks Inc., Natick, MA 01760, USA) was used to carry out principal component analysis (Zitko 1994).

RESULTS

Hatching began on January 27, 1999, and was complete by February 2, 1999. Feeding was started on February 26.

Cumulative mortality in percent of the starting number of individuals exposed to diesel fuel is shown in Fig. 1 and 2. Concentrations of AHs during the treatments are given in Table 1. The large variations in the measured concentrations are caused primarily by oil droplets, present randomly in the extracted samples, particularly in the oil-on-sand samples.

For the controls, a relatively low rate of mortality continued until the end of March, when a plateau at about 20% was reached (Fig. 1). The same stock (Cooke Aquaculture), cultured elsewhere in the hatchery, had a similar mortality pattern. Treatment with 60 µg/L AHs resulted in a slightly elevated mortality rate after hatching, which is probably not significant, since by mid-March it, too, leveled off at about 20%.

Table 1. Concentration of Ahs (µg/L) in the experiments.

	Diesel fuel			Alaskan crude oil	
Date	'60 µg/L'	2%	10%	2% crude	2% weathered crude
14.1	*	49	1200		
18.1	6	2400	12700		
19.1	7				
20.1	66				
21.1	19	535	4300	220	130
24.1	78	1200	2500	17	86
27.1	12	670	12000	7	82
31.1	390	810	1200000	180	6
6.2	8	2800	790000	170	12
9.2	8	23			
22.2	nd	12	780	35	49

*Not measurable, sample contaminated.

Treatment with diesel fuel mixed with sand, however, did markedly affect survival. For the treatments with diesel fuel (Fig. 2), by the time of first feeding, cumulative mortalities were more than 60%, and about 30% for the 2% and 10% treatments, respectively. (Mortality data for the oil-in-sand treatments are not shown after feeding started, since the fry at this stage were mixed freely together in their respective holding tanks, making it impossible to know the treatment history of individuals.). Conspicuous by the order of its effect is the fact that the 2% diesel fuel treatment was considerably more toxic than the 10% treatment.

As can be seen from the UV spectra of the aqueous phases from the model experiment (Fig. 3), the concentration of AHs does not increase linearly with increasing concentration of diesel fuel in the sand; the concentration of AHs, leached from sand containing diesel fuel at 10%, is less than that yielded by sand containing 5% of diesel fuel. With new water (Fig. 4), the concentration of AHs is lower, with the exception of the 1% sand. With subsequent batches of water, the concentration of AHs progressively decreases, but the relative order of diesel fuel-on-sand concentration remains the same (Fig. 5, 6). Different fuel-on-sand concentrations as well as consecutive leachings produce spectra of different shapes as shown in Fig. 7, showing projections of the spectra on the plane of the first two principal components (Zitko 1994).

UV spectra of the WAF of diesel fuel change with time (Fig. 8). The changes are particularly pronounced in the wavelength range from 250 to 270 nm, with the absorbance increasing for 2 h and then decreasing. Spectrum of the hexane extract of the alkaline aqueous phase is similar to that of diesel fuel, but has a weak maximum around 270 nm (Fig. 9). Spectrum of the hexane extract after acidification is different from that of the oil (Fig. 9). On TLC, the former extract shows one spot at about 0.8 of the solvent front. The latter extract has three spots and about 0.3 of the solvent front. All the spots also absorb UV light. According to their properties and extraction conditions, the compounds are likely amines and phenols, respectively.

For the Alaskan oil treatments (Fig. 10), mortality patterns were somewhat similar to those for the diesel fuel-on-sand treatments. By the time of first feeding, mortalities had reached more than 50% and slightly less than 20% for the 2% crude oil and 2% weathered crude oil treatments, respectively. These figures almost certainly would have been higher, had the Alaskan oil treatments not started one week after the diesel fuel treatments.

Plateauing of the rate of mortality over time is suggested by the S-shaped nature of the curves. In the case of the Control and 60 µg/L AH treatments, mortalities became asymptotic well after feeding began, whereas with the oil-on-sand treatments, mortalities leveled off at, or just before, feeding started.

A comparison of the combined mortality of all the treatments immediately after feeding started, 57.2%, with the combined mortality of only 62.6% at the completion of the experiment on May 11, confirms that the direct toxic effects of the oil treatments reached a maximum; very little mortality occurred between the start of feeding on February 26 and the completion of the experiment.

The experiment was continued well after treatments were terminated to investigate the effect on growth, that so characterized the work on pink salmon. Final mean weights and their standard deviations were 1.78 g $\pm$0.48, 1.83 g $\pm$ 0.38, and 1.96 g $\pm$ 0.34, for controls, and 60 µg/L and oil-on-sand treatment, respectively. The average weights are not significantly different.

DISCUSSION

In the work done with pink salmon, AHs derived from Alaskan oil, treated to simulate weathered oil stranded on the Prince William Sound beaches from the *Exxon Valdez* oil spill, caused mortality at a concentration of AHs of only 1 µg/L. In our experiment, AHs derived from samples of Alaskan crude and weathered oil, added to sand at 2%, were toxic to Atlantic salmon eggs and fry, but at much higher concentrations than those reported for pink salmon: from 7 to 220 µg/L for the Alaskan crude oil, and from 6 to 130 µg/L for the weathered oil (Table 1). With respect to exposure levels, exposure timing, and biological sensitivity, our results are similar to those of Billiard et al. (1999), who exposed eggs and fry of rainbow trout *(Oncorhynchus mykiss)* to the AH retene, commonly found in aquatic sediments. In both cases, however, the exposure began shortly after the eyed stage, whereas for pink salmon exposure began after fertilization. It is possible, therefore, that pre-eyed embryos have a greater sensitivity to AHs than later stages of development. The crude oil caused higher mortalities than the weathered oil, as one would expect from the higher AH concentrations, and also from the well-known fact that lower molecular weight AHs are more acutely toxic to aquatic fauna

then those of higher molecular weight. In contrast, the weathered Alaskan oil was more toxic than the Alaskan crude oil to pink salmon. In view of the considerable variations in the concentrations determined by chemical analyses, the apparent difference in toxicities may be the result more of physical conditions of the exposures than biological sensitivities.

There is not enough toxicological information to compare the sensitivity of pink and Atlantic salmon. Surprisingly, in spite of the fact that the toxicity data base AQUIRE (The Chemical Information System) contains the toxicity of 33 chemicals to pink salmon and the toxicity of 109 chemicals to Atlantic salmon, only two organic chemicals, pentachlorophenol and naphthalene, were tested in both species, unfortunately, with different endpoints, thus making comparisons impossible. What can be said is that Atlantic salmon embryos and fry are sensitive to AHs from Alaskan oil at relatively low concentrations, but these AHs seem to be less toxic to Atlantic than to pink salmon.

With diesel fuel, no excess mortality occurred at the nominal AH concentration of 60 µg/L. Significant mortality was observed at higher concentrations, reached in the oil-on-sand treatments. There remains the anomaly of the 2% treatment being more toxic than the 10% treatment. Small 'oil slicks', present on the water surface in the diesel fuel 2% and 10% treatments, were larger and more numerous in the latter, which also contained a more substantial oil phase layer on the sand. Under these conditions, the toxic low molecular weight AHs are more likely to remain in the oil phase, unavailable to the salmon. Model experiments also indicate that higher concentrations of oil on sand may be retaining AHs on the sand. At the same time, the concentration of AHs, as determined by UV spectra of optically homogeneous (not cloudy) aqueous phases, decrease with time. This may indicate the partitioning of AHs between water and an oil phase, whether macroscopic as in the fish experiments, or submicroscopic, as may be postulated from the results of the model experiments. It is interesting to note that naphthalenes from sediments, contaminated by Prudhoe Bay crude oil in the laboratory, were not available to clams (Roesijadi et al. 1978). Unfortunately, the authors did not measure AHs in the aqueous phase during the experiments.

As for the toxicity of diesel fuel as such, the model experiments show that, in addition to hydrocarbons, diesel fuel contains some amines and phenols. The latter tend to be more toxic than hydrocarbons to aquatic fauna. The situation is further complicated by increasing use of hydrogenation of diesel fuel by the industry. Hydrogenation lowers the content of aromatic hydrocarbons, but produces fully or partly hydrogenated naphthalenes (decalines and tetralines, respectively), whose toxicological properties towards aquatic fauna are practically unknown.

The results showing no differences in growth should be viewed with caution. The work by Heintz et al. (1995), showing a negative effect on growth, covered a much longer time period than our experiment. It is possible that 9 weeks after feeding started was not long enough for a long-term, sublethal effect on growth to express itself. Furthermore, it is possible that, consistent with the greater toxicity of Alaskan oil treatments, fry exposed to AHs from this source did have lower growth rates compared to the fry treated with diesel fuel, but statistically this was hidden by the average weight noted earlier having to include individuals from both kinds of treatment.

Whether the results from this work have any practical significance depends, of course, on the concentrations of AHs from diesel fuel, and other sources that could be expected to occur in the salmon's habitat. Model experiments with diesel fuel demonstrate that the partioning of AHs between fuel and water is a complex process, which requires additional studies. The fuel also contains amines and phenols that should be identified and, if necessary, characterized toxicologically.

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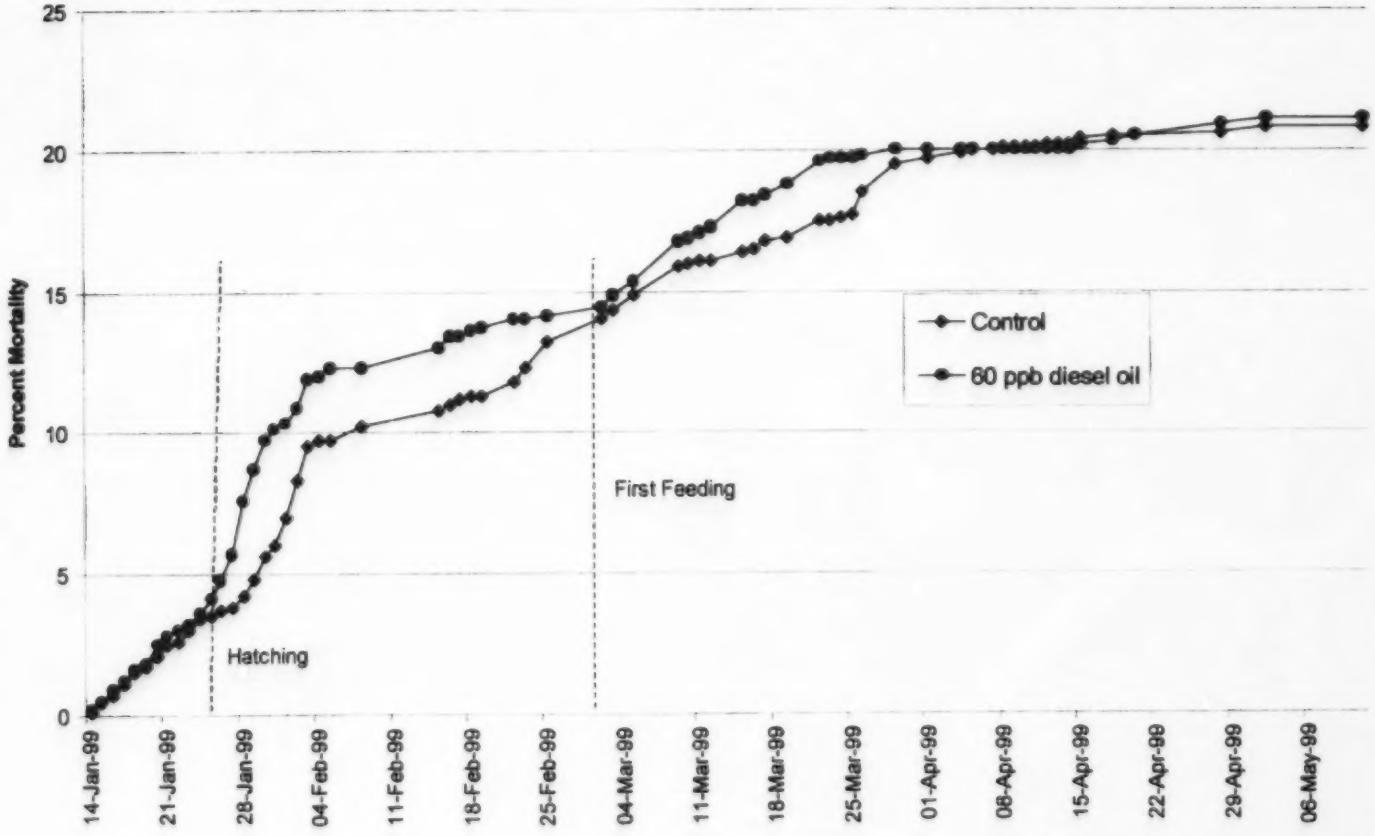

Fig. 1 Cumulative mortality of Atlantic salmon eggs, yolk-sac fry, and first-feeding fry on exposure to diesel fuel at a nominal concentration of 60 µg/L.

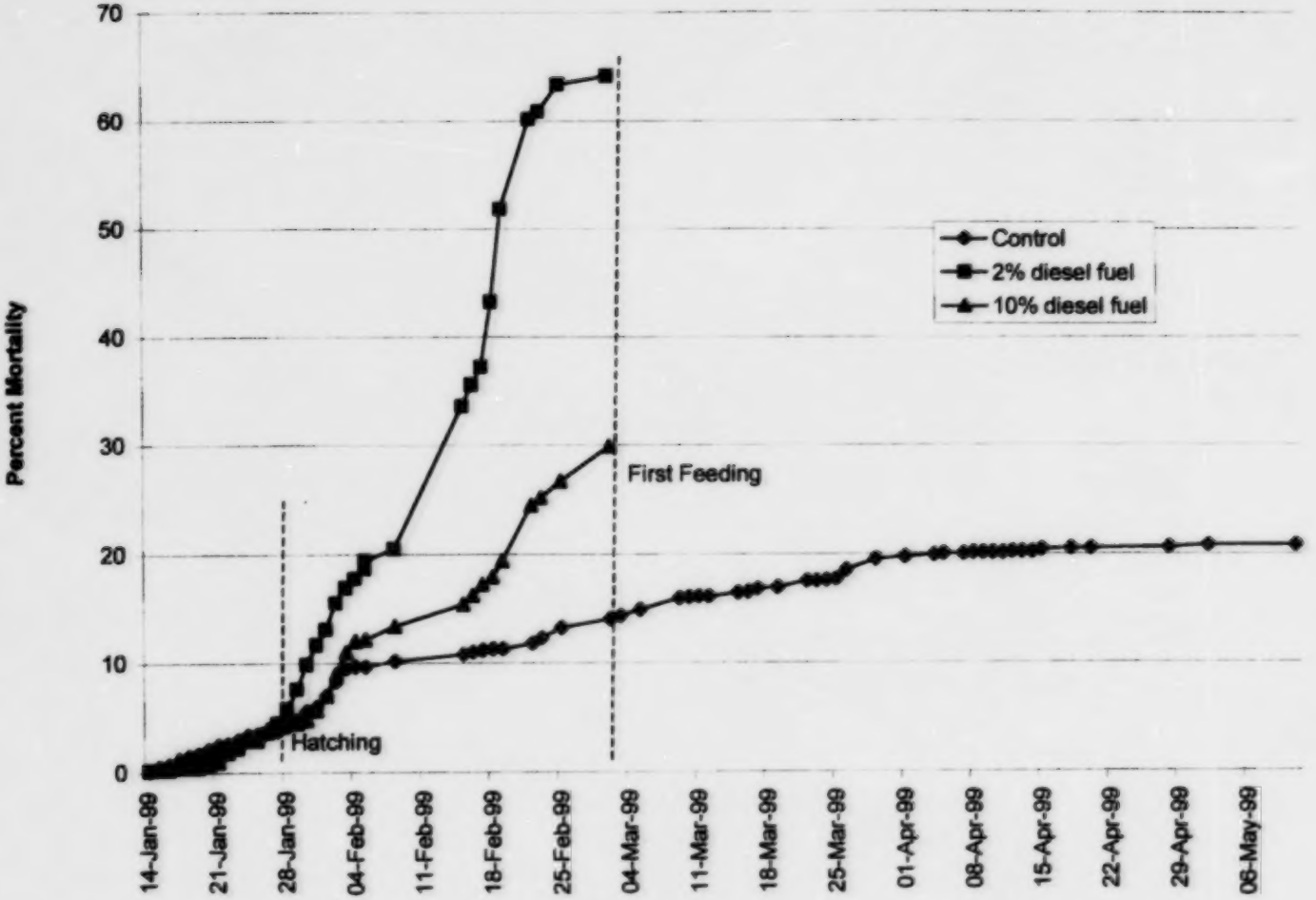

Fig. 2. Cumulative mortality of Atlantic salmon eggs, yolk-sac fry, and first-feeding fry on exposure to sand containing diesel fuel at 2% and 10%.

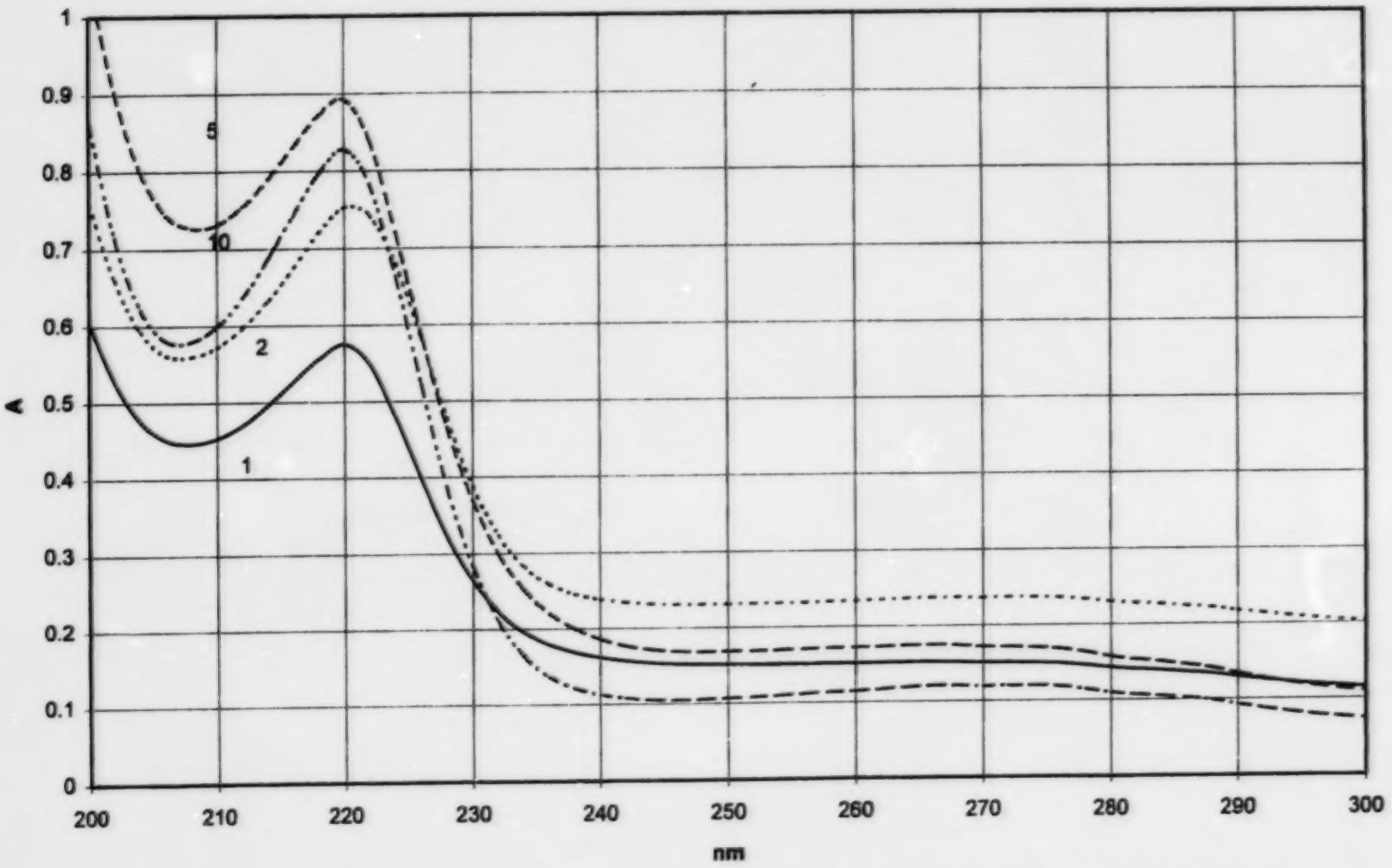

Fig. 3. Ultraviolet (UV) spectra of distilled water (100 mL) equilibrated for 24 h with sand (10 g), contaminated with 1, 2, 5, and 10% of diesel fuel.

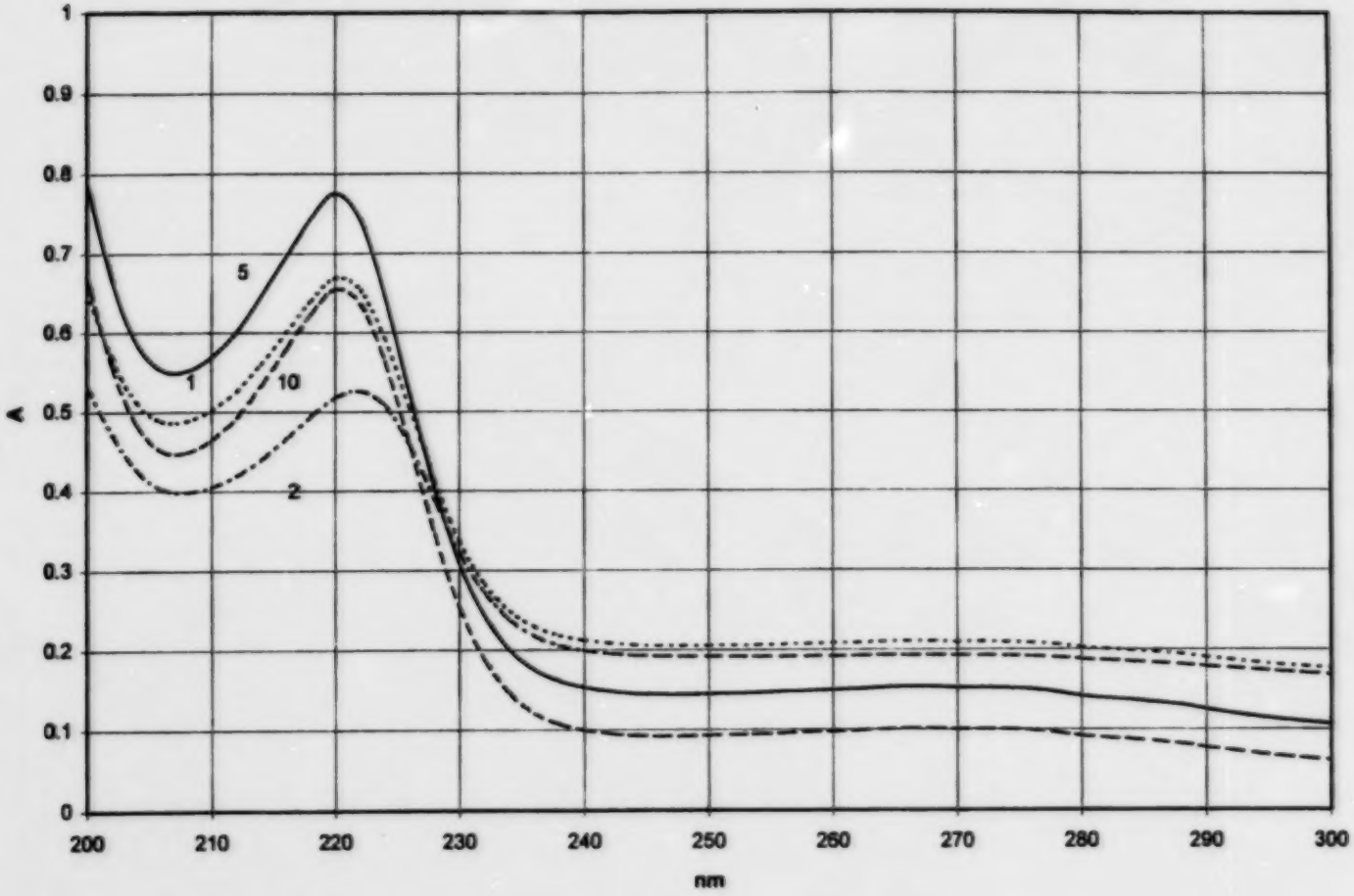

Fig. 4. Ultraviolet (UV) spectra of a 2nd volume of distilled water (100 mL) equilibrated for 24 h with sand (10 g), contaminated with 1, 2, 5, and 10% of diesel fuel.

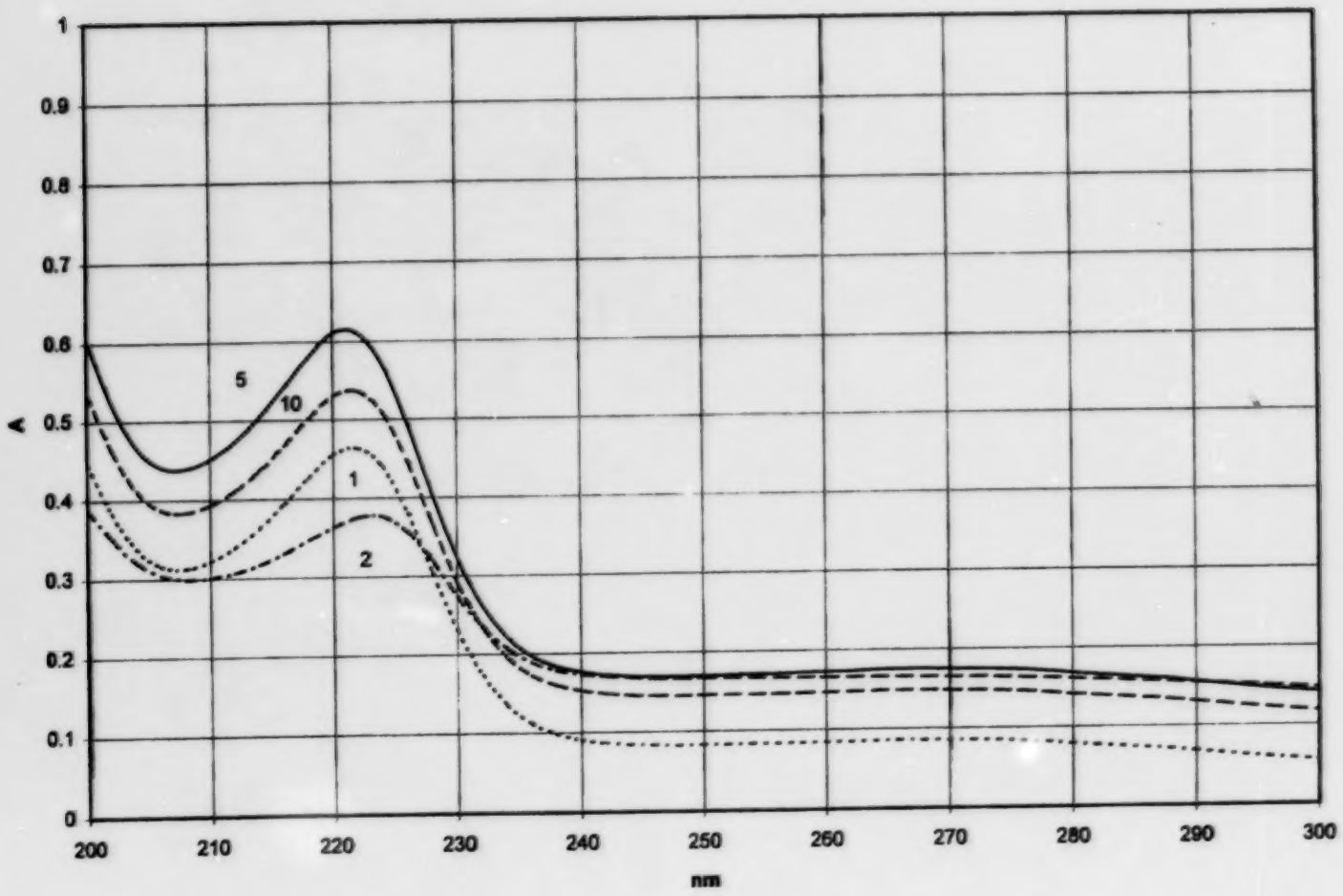

Fig. 5. Ultraviolet (UV) spectra of a 3rd volume of distilled water (100 mL) equilibrated for 24 h with sand (10 g), contaminated with 1, 2, 5, and 10% of diesel fuel.

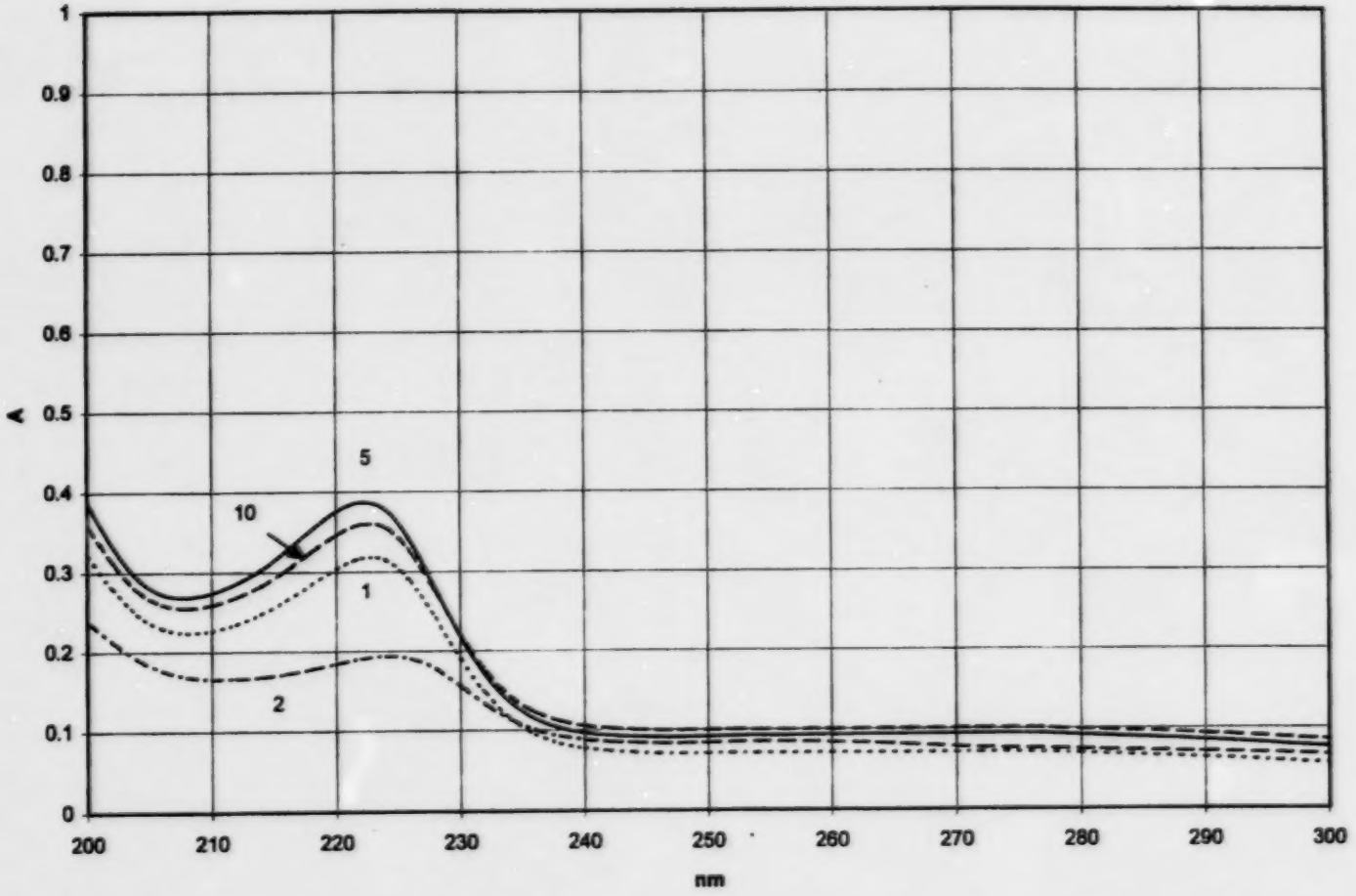

Fig. 6. Ultraviolet (UV) spectra of a 4th volume of distilled water (100 mL) equilibrated for 24 h with sand (10 g), contaminated with 1, 2, 5, and 10% of diesel fuel.

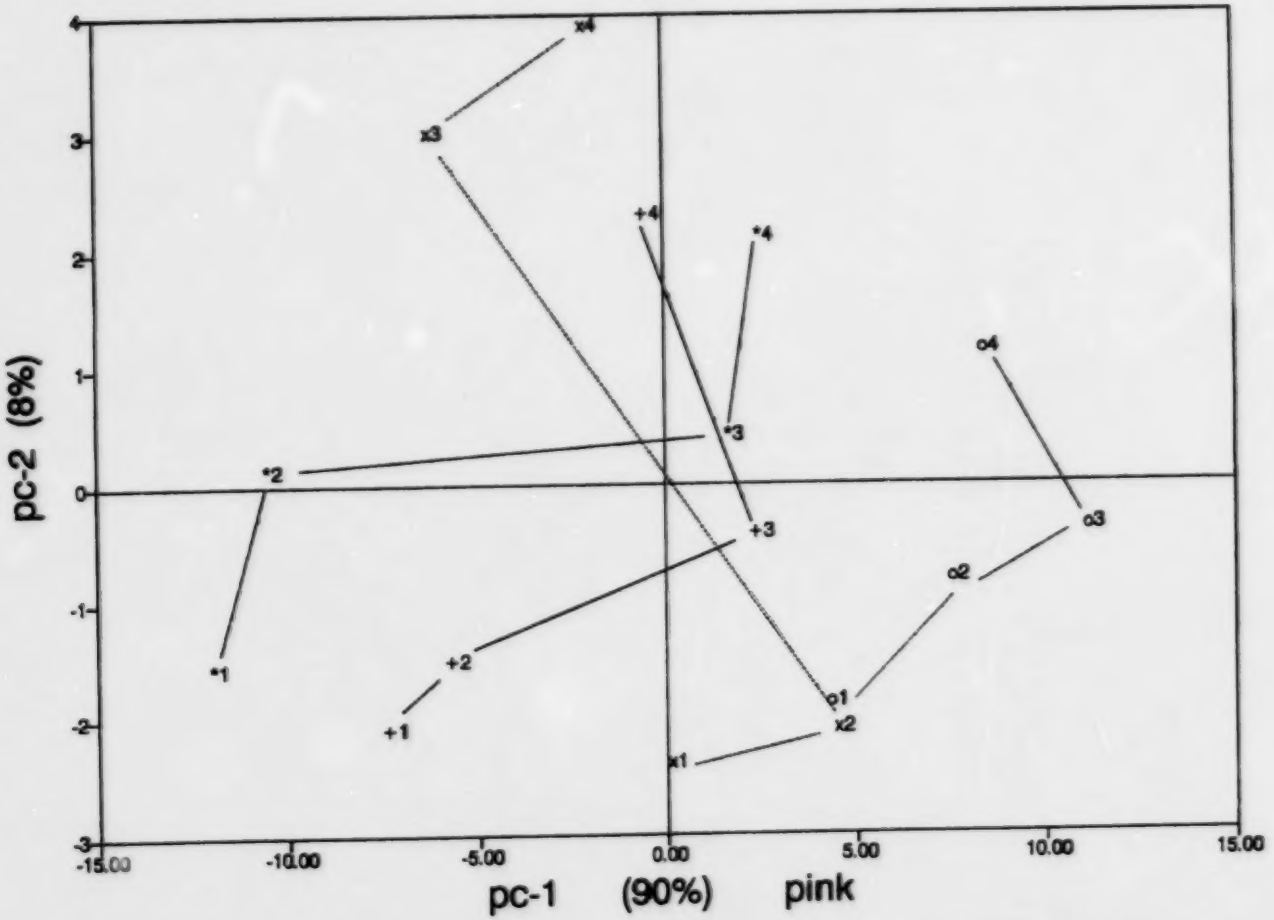

Fig. 7. UV spectra of Figs. 4-7, scaled to unit length and projected by Principal Component Analysis on the plane of the first two principal components. The percentage of the original variance, captured by the principal components is indicated on the axes. In the plot, numbers (1-4) indicate extractions, symbols (x, o, +, and *) refer to sediments, contaminated with 1, 2, 5,and 10% of diesel fuel, respectively.

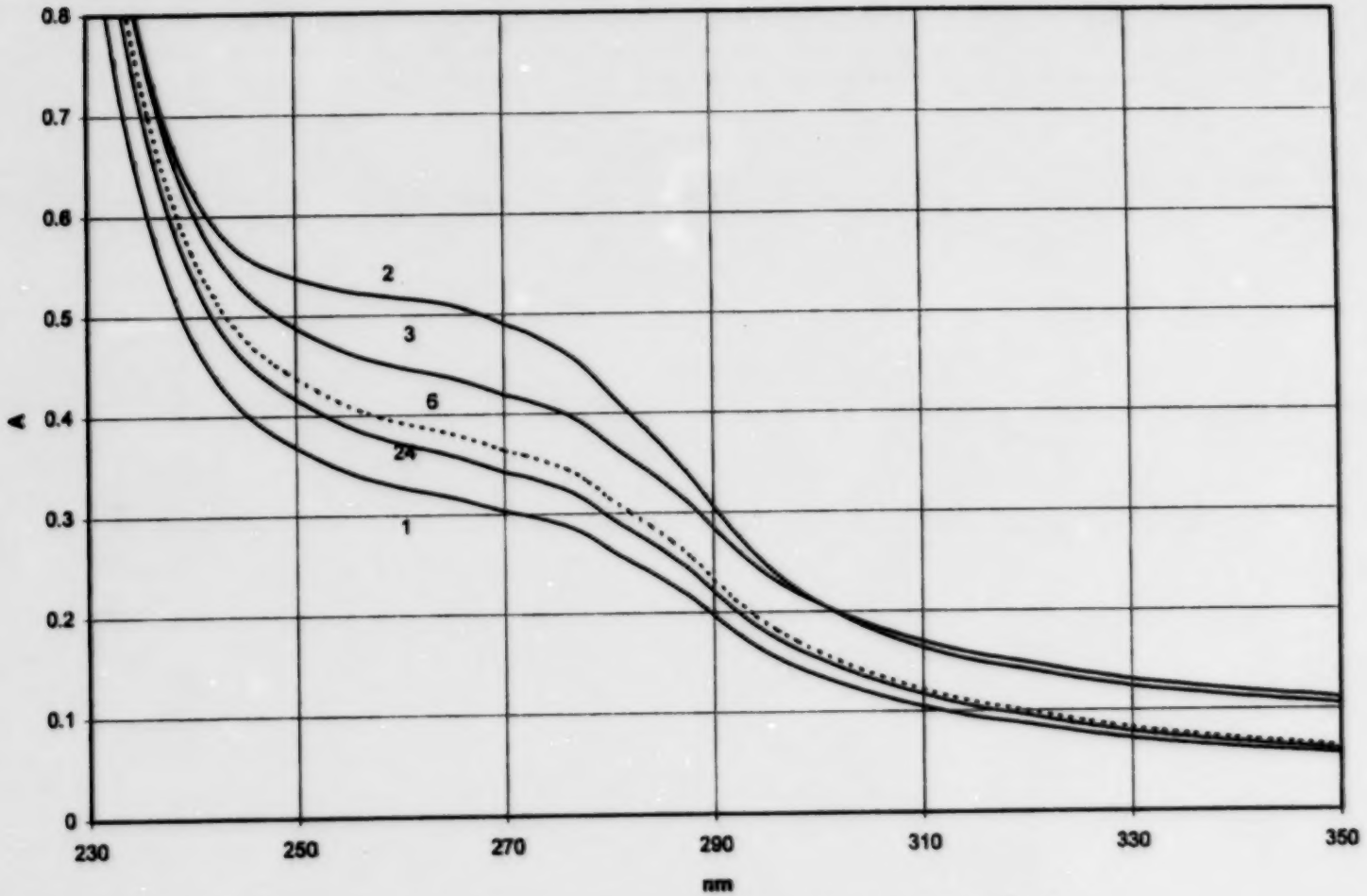

Fig. 8. UV spectra of distilled water (250 mL) in a gentle contact with 50 mL of diesel fuel for 1, 2, 3, 6, and 24 h.

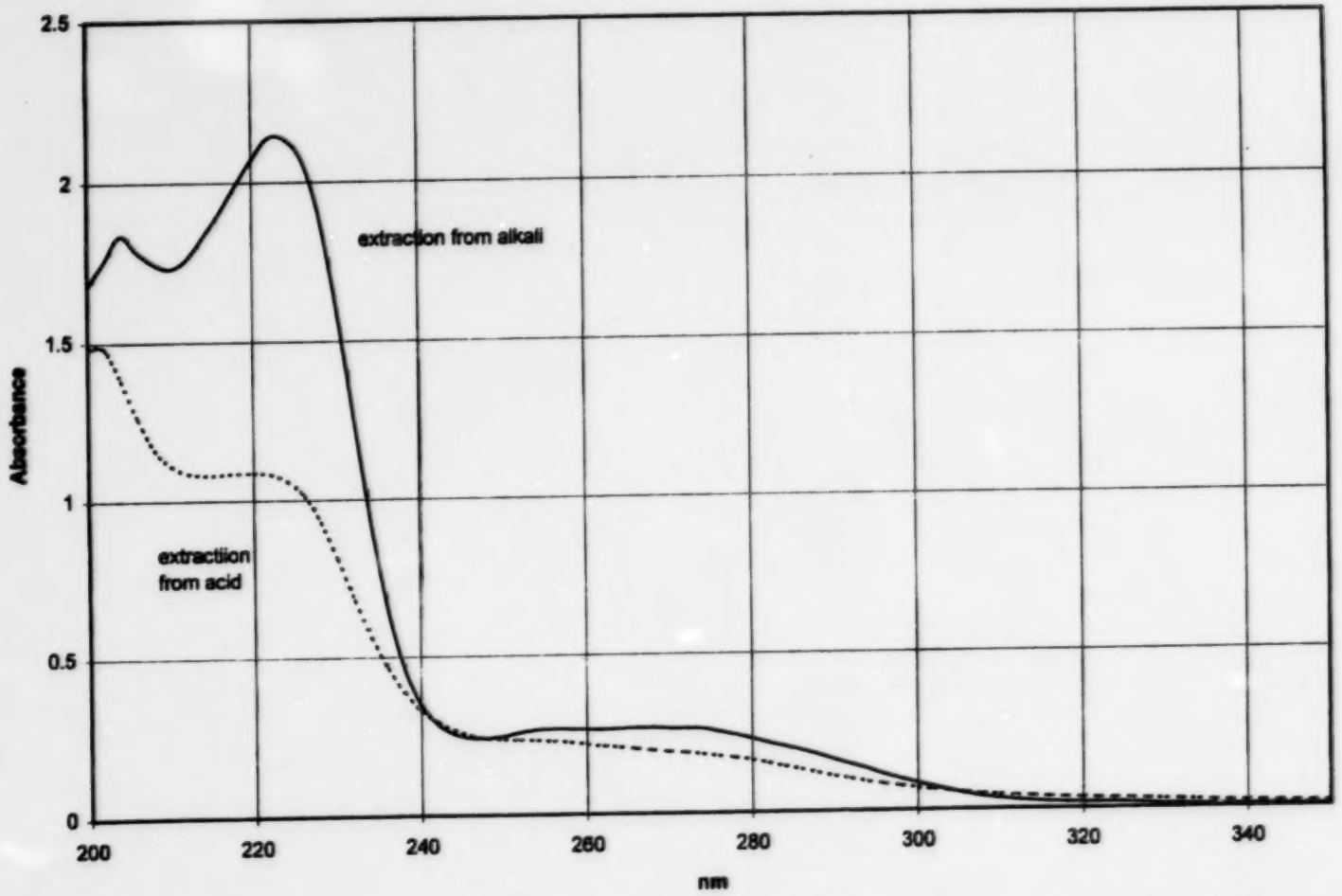

Fig. 9. UV spectra of hexane extracts of water from the 'gentle contact' experiment. The extraction from alkali yields some AHs and amines (absorption maximum and about 270 nm), the extraction from acid yields aromatic acids and phenols. The presence of amines and phenols in these extracts was confirmed by thin layer chromatography and detection with Fast Black K salt (5).

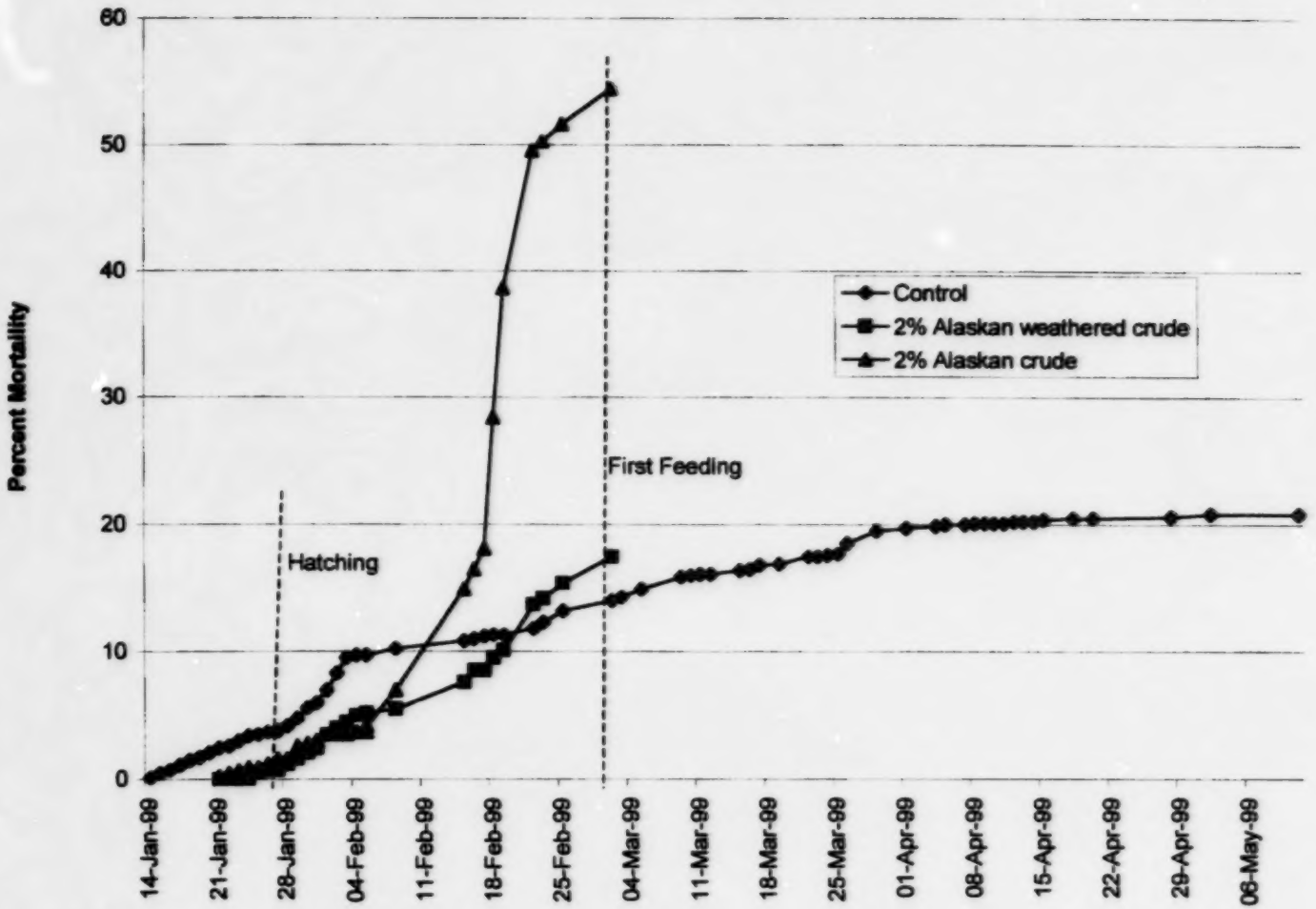

Fig. 10. Cumulative mortality of Atlantic salmon eggs, yolk-sac fry, and first-feeding fry on exposure to sand containing Alaskan crude and artificially weathered crude oil, both at 2%.